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Hypomyelination, memory impairment, and blood–brain barrier permeability in a model of sleep apnea

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ABSTRACT

We investigated the effect of intermittent hypoxia, mimicking sleep apnea, on axonal integrity, blood–brain barrier permeability, and cognitive function of mice. Forty-seven C57BL mice were exposed to intermittent or sham hypoxia, alternating 30 s of progressive hypoxia and 30 s of reoxigenation, during 8 h/day. The axonal integrity in cerebellum was evaluated by transmission electron microscopy. Short- and long-term memories were assessed by novel object recognition test. The levels of endothelin-1 were measured by ELISA. Blood–brain barrier permeability was quantified by Evans Blue dye. After 14 days, animals exposed to intermittent hypoxia showed hypomyelination in cerebellum white matter and higher serum levels of endothelin-1. The short and long-term memories in novel object recognition test was impaired in the group exposed to intermittent hypoxia as compared to controls. Blood–brain barrier permeability was similar between the groups. These results indicated that hypomyelination and impairment of short- and long-term working memories occurred in C57BL mice after 14 days of intermittent hypoxia mimicking sleep apnea.

1. Introduction

Obstructive sleep apnea affects up to one third of the population (Tufik et al., 2010) and is characterized by short, recurrent interruptions in respiratory airflow terminated by an arousal. The breathing instability leads to episodes of intermittent hypoxia (Dempsey et al., 2010).

Patients with sleep apnea show structural and functional disorders of the central nervous system (Lal et al., 2012). Cognitive dysfunctions comprise impairments in attention

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(Sforza et al., 2004), executive function (Lis et al., 2008), and memory (Twigg et al., 2010). In animal models utilizing exposure to intermittent hypoxia to simulate sleep apnea, neuronal death (Zhu et al., 2007) and neurocognitive dysfunctions were observed (Gozal et al., 2001). Our group previously described that cerebellum cells are sensitive to intermittent hypoxia-induced lesions (Baronio et al., 2013).

The mechanisms involved in the association between sleep apnea and cognitive impairment is still unclear. Deficit in executive functioning of children with severe apnea has been linked to decreased neuronal concentration of N-acetyl aspartate, a substrate for lipid and myelin synthesis in oligodendrocytes (Halbower et al., 2006). The brain white matter reduction observed in patients with sleep apnea, including the hypomyelination process in different brain regions (Kim et al., 2013), may play an important role in this relationship. Endothelin-1 has been reported as a key modulator of myelin and axonal damages in animal models of central nervous system injuries (Dos Santos et al., 2007), leading to reactive gliosis and inhibition of neural progenitors proliferation (Li et al., 2010). Moreover, endothelin-1 increases the permeability of the blood–brain barrier in animal models of ischemic stroke (Leung et al., 2009). Sleep apnea increases plasmatic levels of endothelin-1 (Ip et al., 2004). These factors have been studied separately.

We hypothesized that intermittent hypoxia may lead to the impairment in axonal myelination and cognitive performance, as well as to changes in the levels of endothelin-1 and the permeability of the blood-barrier barrier. The present study investigated the effect of 14 days of intermittent hypoxia, mimicking sleep apnea, in serum levels of endothelin-1, blood–brain barrier permeability, axonal integrity, and cognitive function of C57BL mice.

2. Results

2.1. *Endothelin-1 serum levels and blood–brain barrier permeability*

Serum levels of endothelin-1 were significantly higher in the hypoxia group than in the controls (Fig. 1A). No difference in the Evans Blue dye concentrations was observed between groups (Fig. 1B; $P=0.7$; $P=0.8$; $P=0.7$, respectively). No difference between groups was observed in water content of the three regions analyzed (Fig. 1C; $P=0.9$; $P=0.1$; $P=0.6$, respectively).

2.2. *Ultrastructural analysis*

The hypoxia group showed a thinner myelin sheath in cerebellum compared to controls (Fig. 2A and B). Axonal myelin sheath thickness was approximately two-fold larger in control than in hypoxia group animals ($P<0.001$). Axon diameters, however, were similar between control and hypoxia groups ($P=0.9$). The ratio of axon diameter to fiber diameter (*g*-ratio) was significantly higher in animals exposed to hypoxia ($P<0.015$; Fig. 2C), indicating hypomyelination.

2.2.1. *Working and recognition memories*

Fig. 3 shows scores of the animals in the novel object recognition test. Total time exploring both objects was similar between control and hypoxia groups (Fig. 3A). During test 1, the ability to discriminate the two objects was impaired in the hypoxia group, indicated by negative values of discrimination and discrimination index as compared to positive values in the control group (Fig. 3B and E). Hypoxia animals explored the familiar object with more frequency and duration than control group during test 1 (Fig. 3C and D). The information retention and recognition memory were also worse in animals exposed to intermittent hypoxia, indicated by recognition index values lower than 50% (Fig. 3F). The controls presented recognition index above 50%. These results suggest impairment of short-term memory in the hypoxia group.

In the first and second tests, the hypoxia group showed reduced discrimination of novel and familiar objects. These animals spent significantly more time exploring the familiar object, and with more frequency, than the control group (Fig. 3C and D). Discrimination parameters were significantly higher in the control group, indicating the normal preference for novelty (Fig. 3E). Recognition index was also lower in animals exposed to intermittent hypoxia (Fig. 3F), indicating impaired long-term memory.

In the univariate general linear model, controlling for performance in the first test, no significant differences were observed in any variable of the novel object recognition test in the second test ($P>0.05$).

3. Discussion

The findings of the present study comprised the hypomyelination in cerebellum, impairment in short- and long-term memory, and increased levels of endothelin-1 in mice exposed to 14 days of intermittent hypoxia. Furthermore, this is the first study to evaluate the blood–brain permeability in a murine model of sleep apnea.

Cai et al. (2012) reported hypomyelination in a mice model of infantile sleep apnea. They found a reduction in proportion of myelinated fibers in the external capsule, striatum, fornix, and cerebellum. Moreover, the myelin sheath thickness/axon size was significantly lower in the animals exposed to intermittent hypoxia, suggesting a process of demyelination. One noteworthy finding of our study is the extension of the hypomyelination observed after 14 days only of intermittent hypoxia. In studies about axon myelination, chronic intermittent hypoxia protocols are often applied, ranging from 9 days to 4 weeks of exposition (Kanaan et al., 2006; Cai et al., 2012). Although the half-life of some myelin components is around one month in the normal mouse brain (Ando et al., 2003) the response to injury may lead to rapid myelin degeneration. After a crush-induced lesion, myelin disappears in three days (Goodrum et al., 1994) and after an inflammatory insult, myelin disappears in a few days (Jeong et al., 2013).

Imaging techniques have detected brain damage in humans with sleep apnea. These patients show a reduction of the white matter in regions with important neural

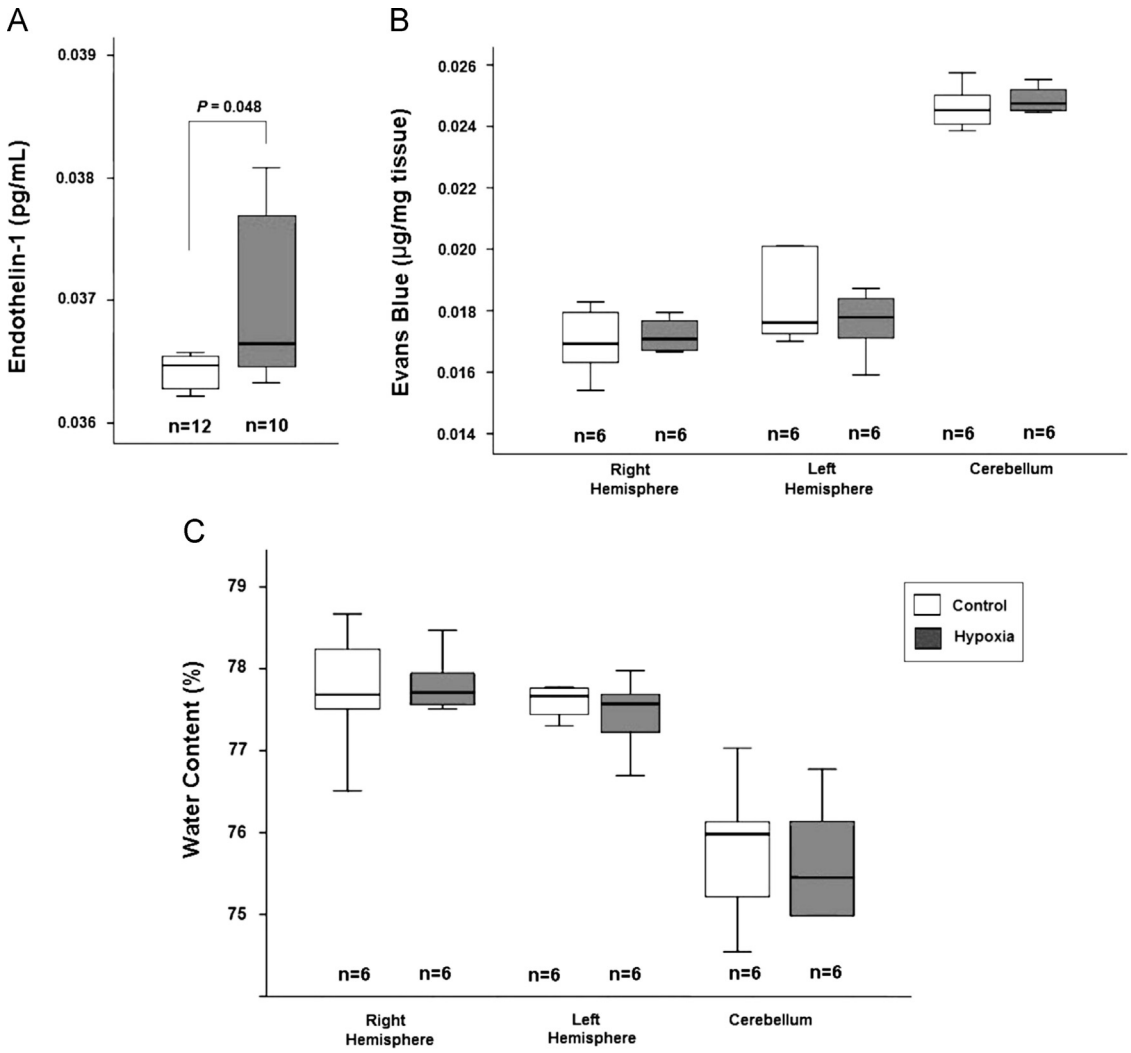

Fig. 1 – (A) Serum levels of endothelin-1 in control and hypoxia animals. (B) Albumin-Evans Blue dye concentrations in cerebral hemispheres and cerebellum of control and hypoxia animals. (C) Water content in cerebral hemispheres and cerebellum of control and hypoxia groups. Data are expressed as median and interquartile range.

connections to hippocampus and amygdala (Macey et al., 2008). This could explain the neurocognitive dysfunctions described in sleep apnea.

Endothelin-1 was higher in the hypoxia group. This peptide and its receptors are widely expressed in different cellular types in central nervous system, including endothelial cells (Kallakuri et al., 2010) and astrocytes (Blomstrand et al., 2004). White matter injury is associated with increase of endothelin-1 levels, with a possible role in the reactive gliosis (Castañeda et al., 2011). This process promotes an inflammatory response characterized by polymorphonuclear and mononuclear leukocytes recruitment to the glial scar (Souza-Rodrigues et al., 2008). Gadea et al. (2008) demonstrated that the activation of endothelin-1 receptors in astrocytes promotes reactive astrogliosis after focal demyelination of corpus callosum. Our group previously demonstrated that 35 days of intermittent hypoxia induces reactive gliosis, astrocytes cell bodies hypertrophy, and neuronal damage in cerebellum, indicated by GFAP and S100B immunoreactivities (Baronio et al., 2013). Furthermore, endothelin-1 levels are also elevated in patients with multiple sclerosis, an inflammatory demyelinating disease of the central nervous system (Haufschild et al., 2001). Thus, endothelin-1 may be involved in the present finding of hypomyelination.

Our results regarding hypomyelination are consistent with the findings of working memory performances. Cerebellum is particularly sensitive to hypoxia and participates in the neural network of consolidation and retrieval of working memory (Stoodley, 2012). Our findings in novel object recognition test indicate short- and long-term memory impairment in hypoxia group, which is in agreement with previous studies. Canessa et al. (2011) reported deficits in short- and long-term memories associated with focal reductions in gray matter volume in hippocampus, parietal cortex, and superior frontal gyrus in patients with severe obstructive sleep apnea. Deficits in spatial working memory in water maze protocol were observed in rodents exposed to intermittent hypoxia (Li et al., 2011). However, it has been suggested that a minimum duration of exposition to intermittent hypoxia is necessary to cause detectable memory impairment. Ward et al. (2009) demonstrated that 24 h of intermittent hypoxia before or after the acquisition training had no significant effect on the

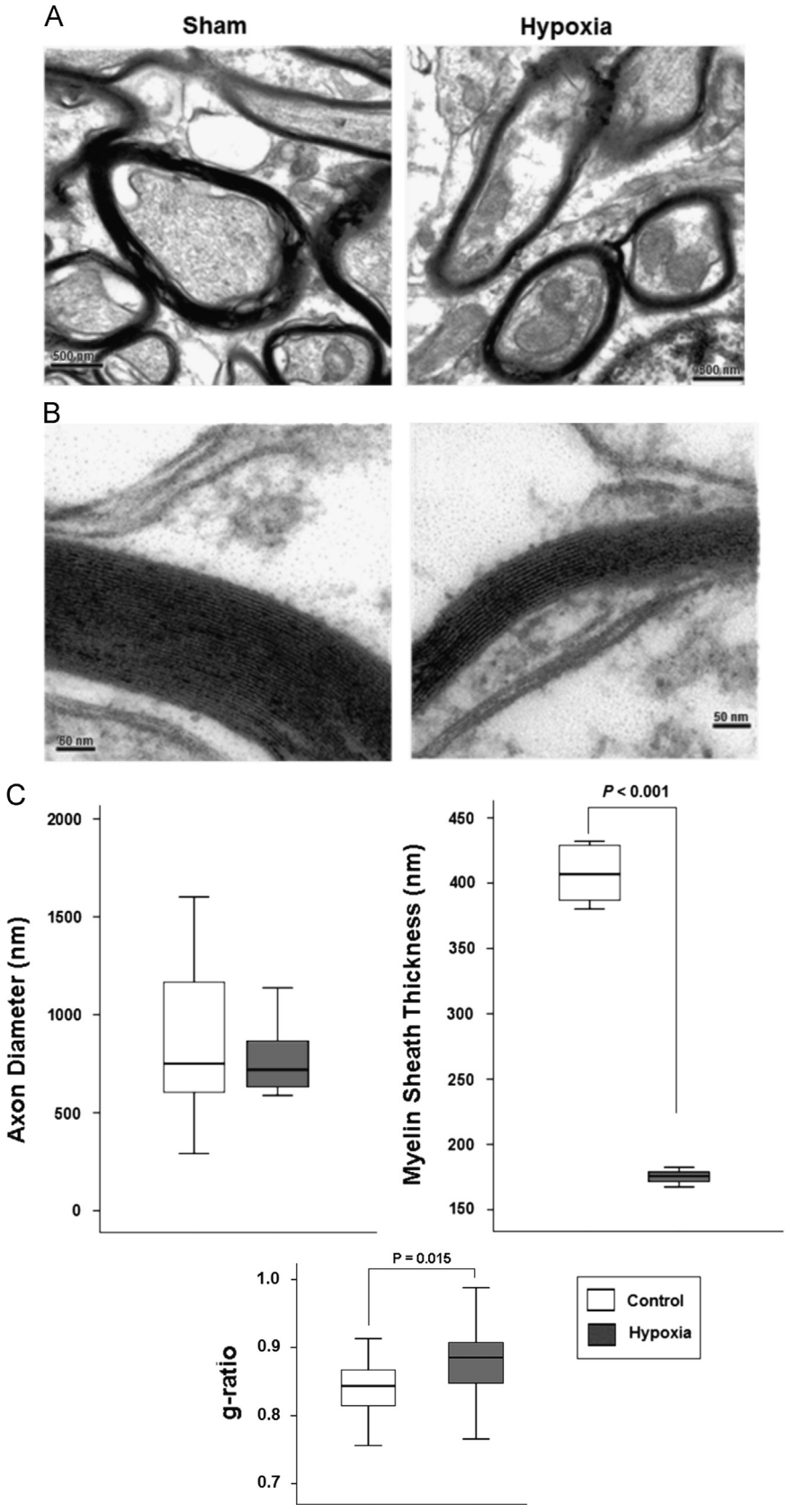

Fig. 2 – Ultrastructural analysis of axonal myelin sheath in cerebellum in hypoxia and control groups. (A) Axons wrapped by myelin (scale 500 nm) in both groups. (B) Images employed for the measurement of myelin sheath thickness (scale 50 nm). (C) Box-plots of axon diameter, myelin thickness, and g-ratio showing the significance. Data are expressed as median and interquartile range.

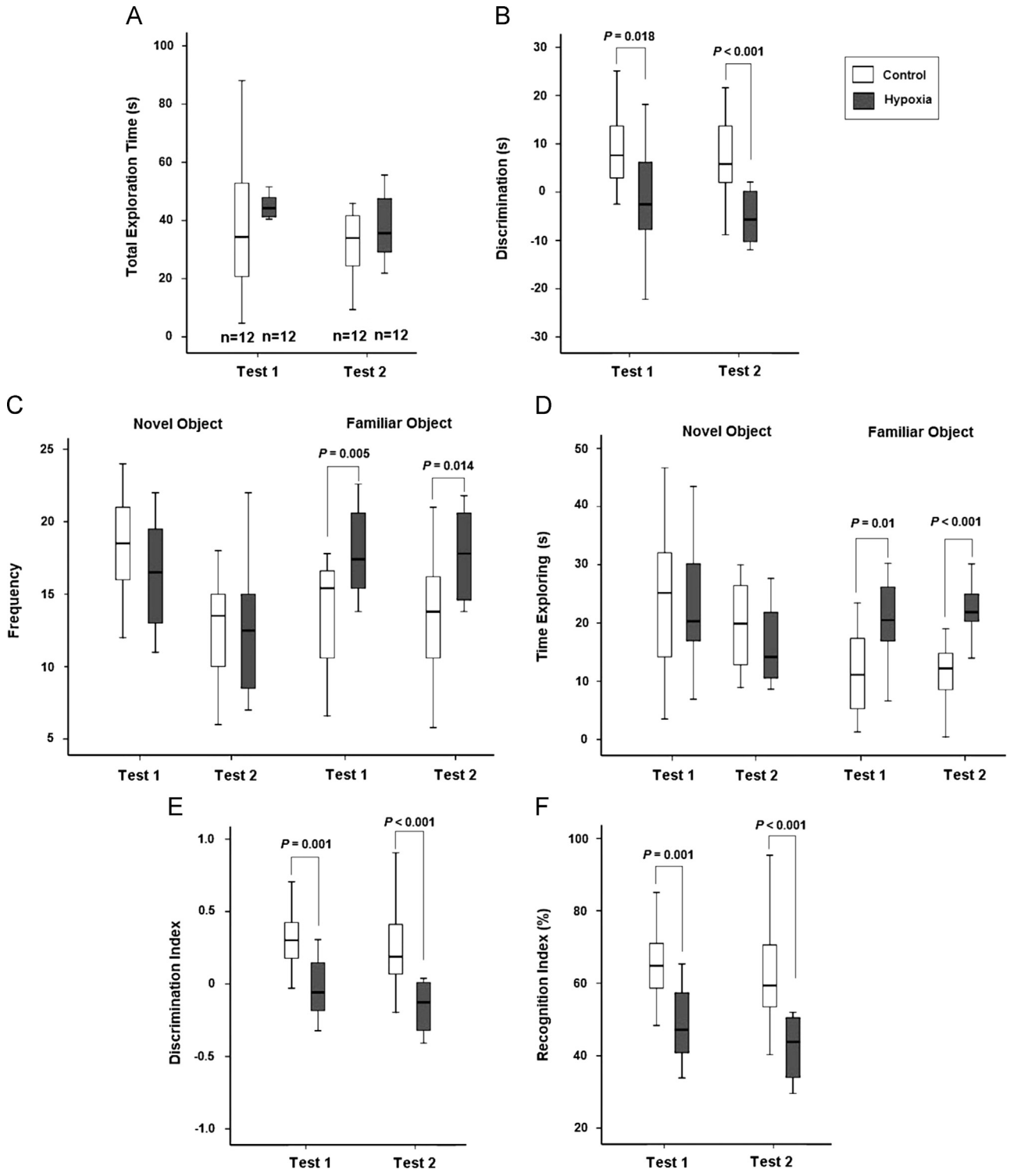

Fig. 3 – Performance in novel object recognition test in control and hypoxia groups. (A) Total time exploring both objects during tests 1 and 2. (B) Differences between time exploring the novel object and time exploring the familiar object during tests 1 and 2. (C) Frequencies exploring novel and familiar objects during tests 1 and 2. (D) Time spent exploring the novel and the familiar object during tests 1 and 2. (E,F) Values of discrimination and recognition indexes, respectively. Data are expressed as median and interquartile range.

memory acquisition and retention process of rats during the water maze evaluation. Memory impairment was only observed in animals submitted to 3 days of intermittent hypoxia.

The novel object recognition test evaluates the working memory in rodents (Ennaceur and Delacour, 1988). In comparison to other memory protocols, the novel object recognition test has some advantages. Firstly, this task is based on

the animal preference for novelty which is a natural and expected behavior (Ennaceur and Delacour, 1988 Antunes and Biala, 2012). Moreover, the novel object recognition test does not demand an aversive stimulation or punishment (light, water, and electric shock) (Silvers et al., 2007), reducing the stressful conditions to which the animals are subjected.

Lim and Pack (2014) suggested that obstructive sleep apnea may cause a disruption of the blood–brain barrier via molecular responses activated by intermittent hypoxia, leading to microenvironment alteration and cognitive impairment. We hypothesized that endothelin-1 could modulate this mechanism of intermittent hypoxia inducing cognitive dysfunctions by increasing the blood–brain barrier permeability. The present study was the first to investigate the effect of intermittent hypoxia exposure, mimicking the obstructive sleep apnea, in the blood–brain barrier permeability. However, the anticipated association between increased endothelin-1 and blood–brain barrier breakdown was not observed. A possible explanation for this result is the downregulation of endothelin-1 receptors in cerebral blood vessels during intermittent hypoxia expositions (Allahdadi et al., 2005). Although intermittent hypoxia may increase serum levels of endothelin-1, a reciprocal reduction in the expression of the endothelin-1 receptors in the blood vessels walls may occur, downregulating vascular sensitivity to the peptide. Furthermore, we performed a global assessment of blood–brain barrier breakdown with Evans Blue dye assessment which could underestimate the focal effect of intermittent hypoxia on blood–brain barrier permeability.

In mice, intermittent hypoxia increases the brain water content after 35 days of exposure (Baronio et al., 2013). In the present study, utilizing an intermittent hypoxia protocol during 14 days, no alterations of water content in the cerebral hemispheres and cerebellum were observed. Periods of intermittent hypoxia exposure longer than 14 days may be necessary to alter the blood–brain barrier permeability and lead to fluid accumulation.

Limitations of the present protocol include lack of interventions aiming to reverse the structural and cognitive impairments caused by the intermittent hypoxia, such as to include a recovery period of sustained normoxia after the protocol of sleep apnea. The use of animals with endothelin-1 gene knockout, endothelin-1 receptors blockers, and measurement of the endothelin-1 receptors mRNA expression, could establish a better causal association between sleep apnea and its repercussions. The ultrastructural alterations after the intermittent hypoxia exposition were only investigated in cerebellum. A larger number of brain regions should be further assessed for myelin content, especially the CA1 region of the hippocampus whose pyramidal neurons activation is associated with the consolidation of recognition memory (Pihlajamäki et al., 2004). In addition, EEG recording during the intermittent hypoxia exposition would allow gauging the extent of sleep disruption. Sleep fragmentation of a sizable magnitude could explain, at least in part, the cognitive decline in the intermittent hypoxia group. Further research is necessary to establish the relative participation of each one of the two main sleep apnea consequences: arousals and intermittent hypoxia.

These results are hypotheses generating. We firstly hypothesized that endothelin-1, by promoting white matter injury and increasing the permeability of the blood–brain barrier, play a role in the memory impairment. In the present study, we observed extensive hypomyelinated axons in the cerebellum white matter of animals exposed to intermittent hypoxia. Our findings also indicate no blood–brain barrier breakdown, although an augmented endothelin-1 serum level was observed. Further research on the mechanisms of endothelin-1 response to intermittent hypoxia and its relationship with the hypomyelination process are warranted.

4. Experimental procedure

4.1. *Animals*

Fourty-seven and two-month-old male C57BL mice, weighing 23.0±1.8 g, were purchased from FEPPS (Porto Alegre, Brazil) and housed in groups of six animals in polypropylene cages at temperatures of 22±1 °C in 12:12-hour light–dark cycle, receiving food and water ad libitum, under the supervision of a veterinarian in the animal experimentation unit at the Hospital de Clínicas (HCPA; Porto Alegre, Brazil). The experimental protocols were designed according with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and approved by the institutional animal care committee, Hospital de Clínicas de Porto Alegre (CEUA/HCPA #2011/11-0499).

4.1.1. *Intermittent hypoxia protocol*

As described before (Halbower et al., 2006; da Rosa et al., 2012; Martinez et al., 2010), 23 animals were exposed to 14 days of intermittent hypoxia (hypoxia group), during 8 h/day from 9:00 a.m. to 5 p.m. Animals were submitted to 30 s of progressive hypoxia with nitrogen gas, reducing the fraction of inspired oxygen (FiO_2) from 21% to approximately 6±1%, followed by 30 s of reoxygenation with room air, restoring the FiO_2 to 21%. The animals were exposed to 8 h of IH daily, totaling 480 cycles of hypoxia/reoxygenation, equivalent to an apnea index of 60/h. The other 24 animals were submitted to sham intermittent hypoxia (control group). This group was housed under the same conditions as the hypoxia group, without nitrogen gas insufflation.

4.1.2. *Endothelin-1 measurement*

Blood samples were collected at day 14 of intermittent hypoxia in 22 animals (control $n=12$; hypoxia $n=10$). Serum levels of endothelin-1 were measured by enzyme-linked immunosorbent assay (ELISA). The determinations were made in duplicate with a mean coefficient of variation of 7.3%. The procedures followed the manufacturer's instructions (Enzo Life Sciences; Farmingdale, NY, USA).

4.1.3. *Evans Blue extravasation*

The quantification of Evans Blue dye was done as reported by Huang et al. (2012) in C57bl mice. After 14 days of IH exposure, the animals received 0.25 mL of 4% solution of Evans Blue intraperitoneally. The solution was allowed to be absorbed

and circulate during 2 h and, after this period, the mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally.

Animals were transcardially perfused with saline injected in the left ventricle with flow rate of 10 mL/min (Infusomat® compact, B.Braun, Brazil) until the color of the liquid observed at the splancnic vessels confirmed the complete removal of the Evans Blue from the circulation. Brains were immediately dissected in cerebellum, right, and left cerebral hemispheres. The samples were weighed and immediately frozen in liquid nitrogen.

The samples were homogenized in 1 mL of phosphate buffered saline (PBS) pH 7.2, sonicated, and centrifuged for 30 min at 12,000 rcf. The 400 μL collected from supernatant were added to 400 μL of 50% trichloroacetic acid. After 24 h at 4 °C, the samples were re-centrifuged during 30 min at 12,000 rcf. The concentration of Evans Blue dye was measured in the supernatant of the samples with a microplate reader (Anthos Zenyth 200rt, Instrulab, Brazil) at 610 nm. Comparing the data against a standard curve, with dye concentrations ranging from 0.12 to 1.95 μg/mL, the level of Evans Blue extravasation was calculated by linear regression. The results were presented as micrograms of albumin-Evans Blue/milligram of brain tissue.

4.1.4. *Brain water content measurement*

After 14 days of intermittent hypoxia, the water content was measured in cerebellum, left and right cerebral hemispheres to evaluate brain edema formation in 6 animals from each group. The parts of the brain were weighed while wet on an electronic scale (Bel Engineering, Italy) precise for 0.0001 g measurements. After that the samples were placed in an oven at 95 °C during 48 h and re-weighed to determine the dry weight. The water percentage was calculated using the formula water content = wet weight–dry weight/wet weight × 100 (Hara et al., 1996).

4.1.5. *Axon integrity evaluation*

Cerebellum was analyzed by transmission electron microscope at 120 kV (JEM 1200ExII, JEOL, Japan). White matter analysis was performed in two different fields randomly captured from 4 animals. Axon diameters and myelin sheath thickness were quantified using the Image-Pro Express 5.1 software (Media Cybernetics—Bethesda, EUA). The ratio of axon diameter to fiber diameter (*g*-ratio) was also evaluated and the average of 20 measurements from each fiber was utilized.

4.1.6. *Novel object recognition test*

Novel object recognition test, first described by Ennaceur and Delacour (1988), is a non-matching-to-sample task to evaluate preference for novelty, working, and recognition memories. To avoid a learning effect from the repetition of behavioral tests, no baseline measures prior to the experimental protocol were included. Mice were submitted to two consecutive days of habituation at days 11 and 12 of intermittent hypoxia. In the habituation, animals were placed in opaque rectangular cages ($40 \times 25 \times 15$ cm^3) covered with sawdust, without objects, during 15 min/day. In the third experimental day, two identical objects, A1 and A2 ($9 \times 8.5 \times 8.5$ cm^3), were placed in two corners of each cage, 5 cm from the walls. Animals were allowed to explore the objects and the environment for 5 min (training phase). Mice were placed back to home cages during 5 min while the objects A1 and A2 were replaced by one identical object (A3) and one different (B1) located at the same A1 and A2 positions. After the 5 min of memory retention interval, animals were allowed to explore the familiar and the novel objects during 5 more minutes. This period was denominated test phase 1 (T1). Twenty-four hours later, the animals were submitted to test phase 2 (T2), following the same procedure as T1.

All the experimental phases were conducted in a sound-isolated room. The objects and the cages were cleaned with 70% ethanol solution to remove any odors cues. The training phase, T1, and T2 were recorded from a vertical point of view with video camera (Nikon D3100, Japan). Exploration was scored when the animals touched or directed their heads to at least 2 cm from the object. Frequency and time spent exploring the novel (TN) and familiar objects (TF) were analyzed.

The discrimination between novel and familiar objects was evaluated subtracting the TN and TF, [discrimination = (TN − TF)], and calculating the discrimination index (DI) by the following formula: [DI = (TN − TF)/(TN + TF)]. The values of DI can range from −1 to +1. Positive values indicate more time exploring the novel object, while negative ones represent more time exploring the familiar object.

To investigate the information retention by the animals, the recognition index (RI) was calculated as [RI = TN /(TN + TF) × 100]. The ability to recognize the novel object was considered present when the RI was superior to 50%.

5. Statistical analyses

Non-normally distributed data were analyzed using Mann–Whitney's *U* test and expressed as median and interquartile range. The results of the novel object recognition test were analyzed by one-way ANOVA. Repeated measures of body weight were analyzed using Generalized Estimating Equations and expressed as mean ± standard deviation. To compare the performances in tests 1 and 2, the Univariate General Linear Model was used. All statistical tests were performed using the SPSS v17.0 software (SPSS Inc., Chicago, IL, USA). Differences with $P < 0.05$ for alpha error were considered significant.

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